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Identification of *cis*-Acting Sequences Responsible for Phorbol Ester Induction of Human Serum Amyloid A Gene Expression via a Nuclear Factor κB-Like Transcription Factor

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We have analyzed the 5′-flanking region of one of the genes coding for the human acute-phase protein, serum amyloid A (SAA). We found that SAA mRNA could be increased fivefold in transfected cells by treatment with phorbol 12-myristate 13-acetate (PMA). To analyze this observation further, we placed a 265-base-pair 5′ SAA fragment upstream of the reporter chloramphenicol acetyltransferase (CAT) gene and transfected this construct into HeLa cells. PMA treatment of these transient transfectants resulted in increased CAT expression. Nuclear proteins from PMA-treated HeLa cells bound to this DNA fragment, and methylation interference analysis showed that the binding was specific to the sequence GGGACTTTCC (between −82 and −91), a sequence previously described by R. Sen and D. Baltimore (Cell 46:705–716, 1986) as the binding site for the nuclear factor NFκB. In a cotransfection competition experiment, we could abolish PMA-induced CAT activity by using cloned human immunodeficiency virus long-terminal-repeat DNA containing the NFκB-binding sequence. The same long-terminal-repeat DNA containing mutant NFκB-binding sequences (G. Nabel and D. Baltimore, Nature [London] 326:711–713, 1987) did not affect CAT expression, which suggested that binding by an NFκB-like factor is required for increased SAA transcription.

Human serum amyloid A (SAA) is a major acute-phase reactant produced mainly by the liver. During periods of inflammation and tissue damage, serum levels of SAA can increase by up to 1,000-fold. SAA is also the precursor peptide of the amyloid A protein subunit of amyloid fibrils in secondary, or reactive, amyloidosis (24). Insoluble fibrils are deposited extracellularly in multiple organs, compromising their normal function. This is a serious complication of chronic or recurrent inflammatory conditions, e.g., juvenile chronic arthritis, in which persistently high serum levels of SAA are found. More than one human SAA gene exist (16, 25, 41), and in mice three active SAA genes and a pseudogene have been described (29, 51). Both human and murine SAA gene expression can be induced by the cytokines interleukin-1 (IL-1) and tumor necrosis factor (39, 49).

We have previously characterized a human SAA gene, SAAg9, and have demonstrated that expression of this gene can be induced by both of these cytokines in transfected mouse L cells (49). How these factors mediate control of gene expression is unknown. To study the mechanism of the regulation of human SAA gene expression, we have characterized the 5′-flanking region of the gene by DNA sequence analysis. Using DNA constructs containing part of 5′-flanking region of SAA upstream of the reporter chloramphenicol acetyltransferase (CAT) gene, we have identified a phorbol ester-inducible enhancer region. We have demonstrated that a phorbol 12-myristate 13-acetate (PMA)-inducible nuclear protein factor(s) binds to the sequence GGGACTTTCC, a sequence first described by Sen and Baltimore (44) to bind the nuclear factor NFκB, and that this binding is required for enhancer activity.

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MATERIALS AND METHODS

DNA sequence analysis. DNA sequence analysis was performed by the chain termination method of Sanger et al. (42). DNA fragments from the 5′-flanking region of the SAA genomic clone, SAAg9 (49), were subcloned into M13mp18 and -mp19 and sequenced by using sequence-specific oligonucleotides generated on an Applied Biosystems automated nucleotide synthesizer.

Plasmid-constructions. A 265-base-pair (bp) *Sau*3A DNA fragment, from the promoter region and 33 bp of the first exon of the human SAA gene, was cloned between the *Bgl*II-*Bam*HI sites of the vector, pTK.CAT3 (31). This construct contains the entire CAT gene, and the thymidine kinase promoter has been replaced with the SAA 5′-flanking region. As a control, the thymidine kinase promoter was deleted from pTK.CAT3 with *Bgl*II-*Bam*HI. The subsequent constructs, OCAT/265 and OCAT, were used to study PMA inducibility conferred on the CAT gene by the SAA promoter region.

The 265-bp promoter fragment was also cloned into the *Bam*HI site of the vector pBluescriptSK, generating plasmid 9-2. This vector contains both T3 and T7 promoters flanking the cloned insert, and it was used to generate both coding- and noncoding-strand cRNA probes used for RNase mapping.

Wild-type ($^{A}_{G}$GGGACTTTCC) and mutant ($^{A}_{G}$CTCACTT TCC) NFκB-binding sequences from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (32), cloned into plasmid pGEM, were kindly provided by G. Nabel for cotransfection-competition studies.

Cell lines and DNA transfections. HeLa cells were cultured in minimal essential medium with 10% heat-inactivated fetal calf serum in 5% CO_2, and mouse L cells were cultured in Dulbecco modified Eagle medium with 10% heat-inactivated

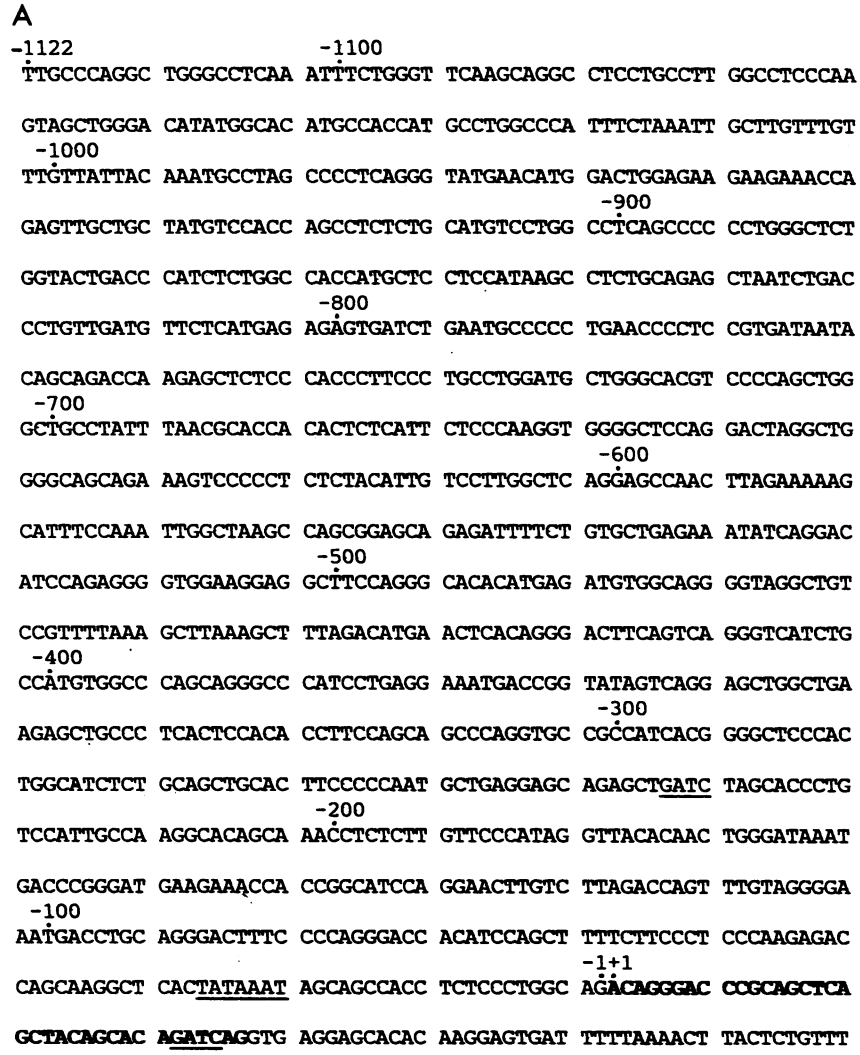

FIG. 1. (A) Nucleotide sequence of the 5'-flanking region of a human SAA gene. The nucleotide sequence of the 5'-flanking sequence, exon 1 (bold face), and part of intron 1 of the SAA gene contained within the genomic clone SAAg9 was determined as described in Materials and Methods. The repeat sequences described in the text are at −54 to −73 and −187 to −209 and at −128 to −142 and −145 to −159 (direct repeats) and at −112 to −130 and −205 to −225 (inverted repeats). TATA box and the *Sau*3A restriction sites used in subsequent cloning of a 265-bp fragment are underlined. (B) Homologies between the SAA 5' region and other 5' regulatory regions. i, Homologies between SAAg9 and IFN-α consensus sequence; iia, homologies between SAAg9 and mouse SAA1 (mSAA1) and SAA2 (mSAA2); iib, similar spatial arrangements of homologous regions of human SAAg9 and mouse SAA1 and 2; iii, locations of sequences homologous to the viral enhancer consensus that binds NFκB (GH, growth hormone; Apo CIII, apolipoprotein CIII); and iv, consensus sequence in the 5'-flanking regions of IL-1-responsive genes (Strom, stromelysin; FB, factor B; Hp, haptoglobin).

fetal calf serum in 5% CO_2. DNA transfections were performed by the calcium phosphate precipitation method (22). OCAT, OCAT/265, and competitor DNAs were cotransfected with 2 μg of plasmid pXGH5 (43), containing the growth hormone gene. At 24 h after transfection, the presence of secreted growth hormone was measured by radioimmunoassay (Allegro human growth hormone assay kit; Nichols Institute) to monitor the efficiency of DNA uptake by cells. The growth hormone assay was subsequently used to normalize the amount of cell extract used in the CAT assay. PMA was added 24 h after transfection, and cell extracts were generated at 28 h.

CAT assays. CAT assays were performed by a modification of the method described by Crabb and Dixon (13), and values were normalized for transfection efficiency as mentioned above. After autoradiography, the acetylated and unacetylated forms of chloramphenicol were quantified by excision and then counting by liquid scintillation.

RNA isolation, RNase mapping, and Northern (RNA) blot analysis. Total RNA was isolated from semiconfluent petri dishes of transfected and nontransfected cells by the guanidinium isothiocyanate total cell lysis method (9), and the RNA was purified by ultracentrifugation through a 5.7 M cesium chloride cushion.

T3 and T7 polymerases were used to generate coding- and noncoding-strand cRNA probes from recombinant 9-2, which contains 236 bp of 5' promoter sequence and 33 bp of the first exon of the SAA gene. These probes were then used for RNase mapping (30, 52). RNA (15 to 30 μg) was annealed with 10^5 cpm of a single-stranded probe at 85°C for 5 min and

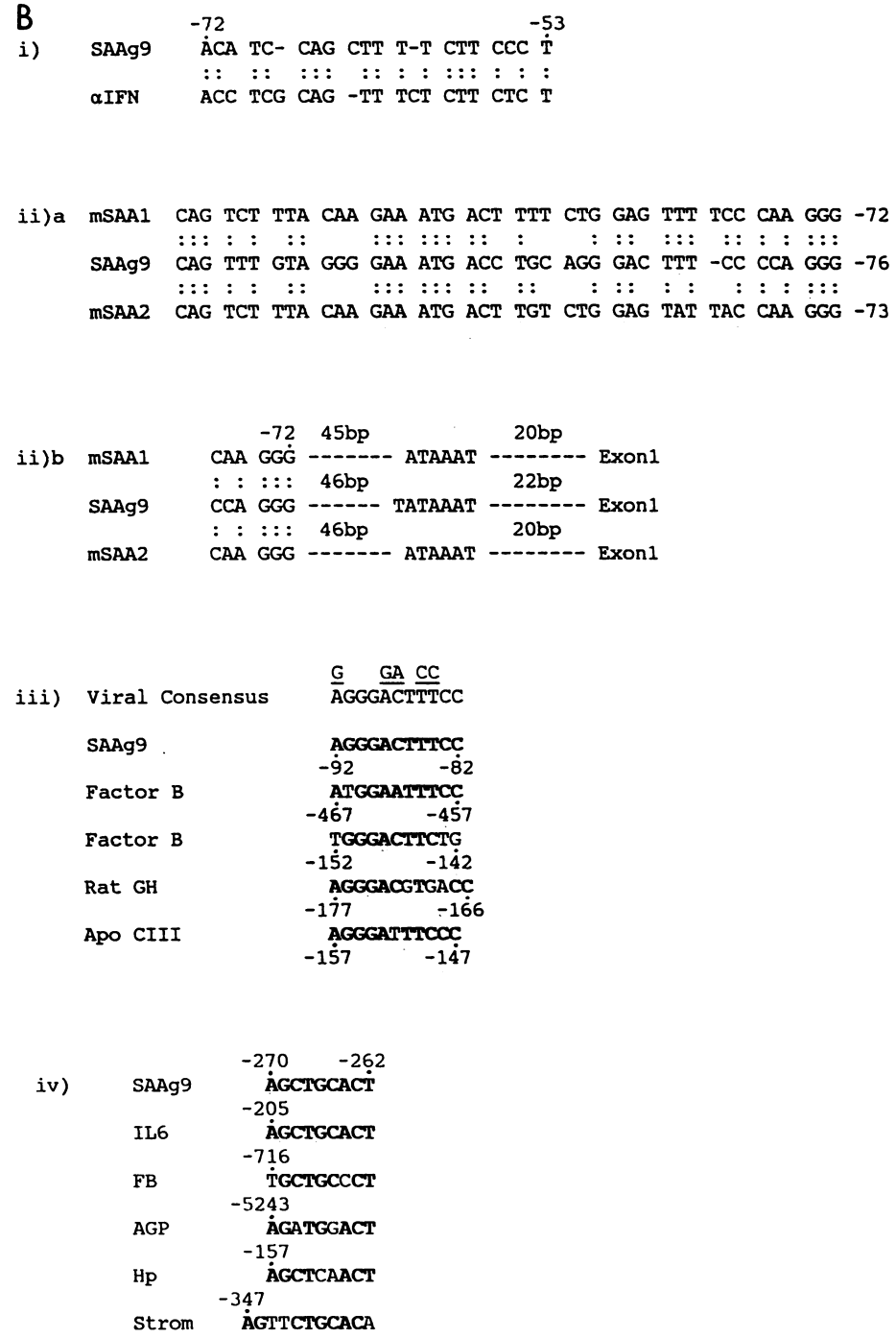

FIG. 1—*Continued*

then allowed to cool overnight to 40°C. Unprotected RNA was then removed by incubation with RNase T_1 at 30°C for 1 h, and the subsequent fragments were separated on a 12% urea–polyacrylamide sequencing gel. Northern blot analysis was performed by electrophoresis of 20 μg of total RNA through 1% (wt/vol) agarose gels containing 0.6% (vol/vol) formaldehyde, transferred to nitrocellulose, and hybridized by using a ^{32}P-labeled SAA-specific cDNA probe, pA1 (46).

Nuclear extract isolation and DNA-binding gel electrophoresis assay. Nuclear protein extracts were generated essentially by the protocol of Dignam et al. (15), and protein concentrations were determined by the BCA-1 assay (Sigma Chemical Co., St. Louis, Mo.).

Analysis of protein-DNA complexes was performed as described by Sen and Baltimore (44). DNA fragments were isolated by electrophoretic transfer onto DE-81 ion-exchange paper, followed by elution in 1.5 M NaCl. DNA-binding reactions (5 μg protein per 10^4-cpm fragment) were carried out at room temperature for 15 to 30 min, with a preincubation of 15 min for unlabeled competitor DNAs. All

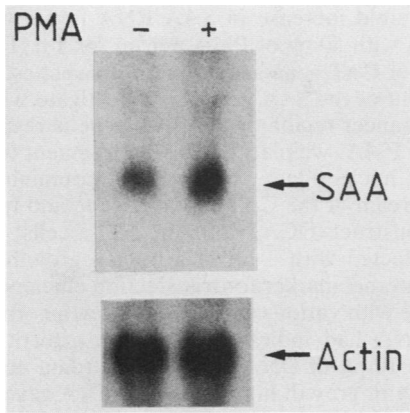

FIG. 2. Northern blot of total RNA from PMA-treated transfected cells. Total RNA was isolated from SAAg9-transfected mouse L cells that were untreated (−) or treated (+) for 4 h with PMA (50 ng/ml) in the absence of fetal calf serum. A 20-μg sample was assayed by Northern blotting, and the filter was probed with ^{32}P-labeled SAA-specific cDNA. After autoradiography, the filter was washed and reprobed with ^{32}P-labeled mouse α-actin cDNA.

reactions were performed in the presence of 3 μg of poly(dI-dC) as nonspecific competitor DNA.

DNA methylation interference assay. DNA methylation interference was assayed essentially as described by Sen and Baltimore (44).

RESULTS

5′-Flanking-region sequence of SAAg9. Sequence analysis of the 5′-flanking region of the SAA gene identified a number of potential regulatory elements (Fig. 1).

First, there is a TATA box (sequence TATAAAT) between nucleotides −23 and −29 (Fig. 1A).

Second, we found homology to alpha interferon (IFN-α) consensus sequence and the 5′ region of the complement factor B gene (Fig. 1B). Between −53 and −72, there is 81% homology to the IFN-α-responsive consensus element found in the IFN-α-inducible HLA and metallothionein genes (18). The factor B gene, another acute-phase gene, contains a similar sequence (50), showing 73% homology to the corresponding region in the SAA gene.

Third, the three mouse SAA genes contain a region of significant homology across 26 nucleotides in their 5′-flanking regions (Fig. 1B; 29). This homology is strongest between the mouse SAA1 and SAA2 genes. The human SAA gene contains a sequence bearing 73% homology to this region across 29 nucleotides, including the 26-nucleotide region of mouse SAA1 and SAA2. This homology is still 69% when the sequence region is extended to 41 nucleotides. Also striking are the similar spatial arrangements of the human and mouse homologies, all starting between nucleotides −72 and −76. However, the homology is only 50% when this region is compared with the corresponding region of mouse SAA3.

Fourth, the 11-nucleotide sequence AGGGACTTTCC, between −82 and −92, is contained within the sequence described above and is identical to the enhancer sequence

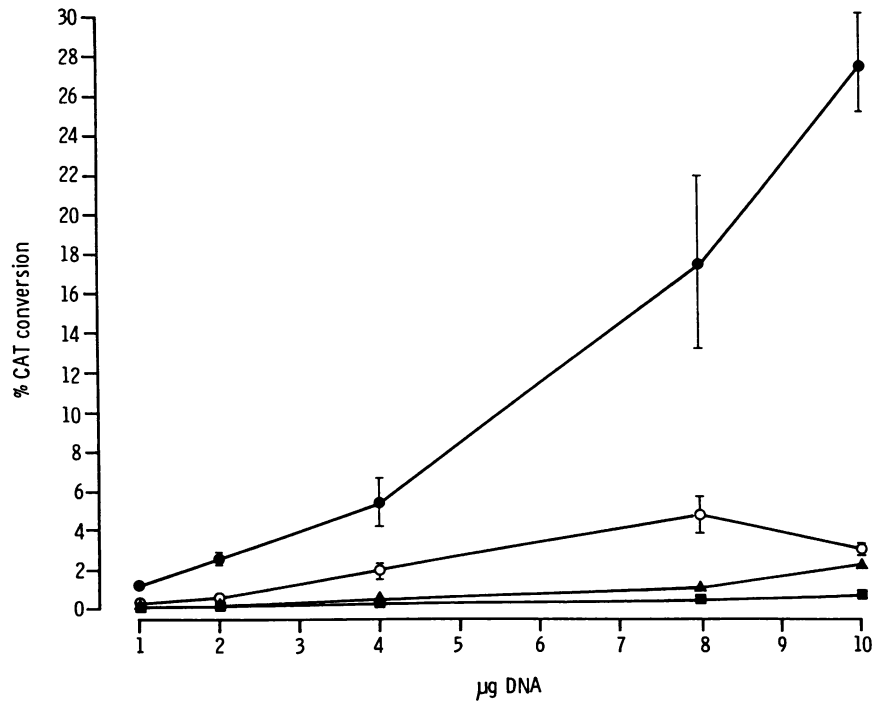

FIG. 3. Dose-response curve of CAT expression after PMA stimulation, using different quantities of test DNA in transfection experiments. Duplicate plates of 10^6 HeLa cells were transfected with OCAT/265 and OCAT. After 24 h, culture medium was assayed for human growth hormone, cells were treated with PMA (50 ng/ml) for 4 h, and the CAT assay was performed. Percent conversion to acetylated forms was normalized to values for growth hormone expression. Symbols: ■, OCAT, no PMA; ▲, OCAT, PMA treated; ○, OCAT/265, no PMA; ●, OCAT/265, PMA treated.

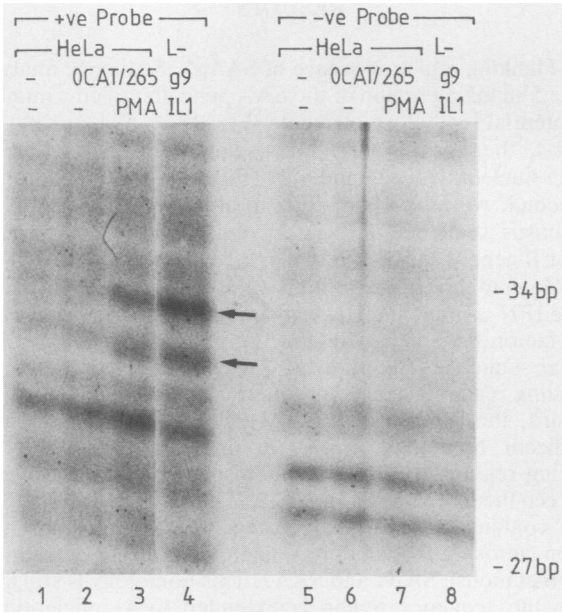

FIG. 4. Mapping of the transcriptional initiation site of the human SAA gene. RNase protection analysis was used to identify the transcriptional start site of the human SAA gene and to determine whether the SAA 265-bp-directed CAT RNA was initiated at the same position. The 265-bp 5′ SAA fragment (Fig. 1A) was subcloned into pBluescript and linearized with *Xba*I or *Eco*RI to allow synthesis of complementary (positive) or noncomplementary (negative) cRNA probes. The ^{35}S-labeled probes were annealed with 30 (lanes 1 to 3 and 5 to 7) and 15 (lanes 4 and 8) μg of total RNA from different transfected cells and treated with RNase T_1 at 30°C for 1 h; the resultant fragments were separated on a 12% polyacrylamide sequencing gel. ^{32}P-labeled nucleotide size markers (shown on the right) were electrophoresed in a parallel track. Arrows indicate protected fragments.

$^{G}_{A}GGG^{GA}_{AC}T^{CC}_{TT}CC$, identified in simian virus 40, human and mouse cytomegaloviruses, HIV-1 LTR, and the immunoglobulin κ light-chain enhancers (28). This sequence has been shown to be a *cis*-acting transcription element responsive to phorbol esters. Two regions in the factor B gene are homologous with the sequence (91 and 72%) (50); regions of homology are also found in the c-*erb*-A-binding site in the growth hormone gene (75%) (21) and the 5′ region of the human apolipoprotein CIII gene (91%) (37).

Fifth, between nucleotides −54 and −225 there are two inverted-repeat regions (21 and 19 bp) and two direct-repeat regions (Fig. 1A). Also, the upstream portion of one of the direct-repeat regions bears 79% homology to a 5′ repeat region of the β-fibrinogen gene, another acute-phase gene (17).

Finally, there is a consensus sequence consisting of 9 bp in the 5′ region of SAA and the IL-1-inducible genes IL-6 (40), stromelysin (37), complement factor B (50), α_1-acid glycoprotein (AGP; 19), and haptoglobin (33) (Fig. 1B).

Control of SAA gene expression by phorbol esters. It was previously shown that the sequence GGGACTTTCC is an enhancer sequence in the immunoglobulin κ light-chain gene and that this sequence is also responsive to phorbol ester induction in non-B cells. To establish whether the SAA gene is inducible with phorbol esters, we treated mouse L cells persistently transfected with the SAA gene SAAg9 with PMA. Analysis of total RNA by Northern blotting demonstrated a fivefold increase in SAA RNA levels when cells were treated with 50 ng of PMA per ml for 4 h (Fig. 2).

Induction of CAT gene activity with phorbol ester by a 5′ DNA fragment of the SAA gene. To investigate whether the potential enhancer region of the SAA gene is responsive to induction by PMA, we placed a 265-bp fragment from the 5′ region (from nucleotides −234 to +31), containing the enhancer, upstream of the CAT reporter gene and transfected this DNA construct (OCAT/265) into HeLa cells. The DNA was cotransfected with 2 μg of a human growth hormone gene as an internal marker for transfection efficiency. Transfections done with various amounts of growth hormone gene (pXGH5) DNA had indicated maximum growth hormone activity with 10 μg of DNA per 10^6 cells (data not shown), although 2 μg of growth hormone gene DNA gave a consistently detectable level of growth hormone. Therefore, all transfection experiments were performed with 10 μg of DNA in total, consisting of various amounts of test DNA, 2 μg of growth hormone DNA, and Bluescribe plasmid DNA used as the carrier.

Incubation of the OCAT/265-transfected cells with PMA for 4 h induced CAT activity approximately 11-fold greater than the level observed in untreated cells. Titration of the amounts of transfected DNA revealed a proportional increase in CAT activity with increasing amounts of DNA (Fig. 3), peaking at 8 μg but then decreasing slightly after this amount. It was also found that the OCAT/265 construct produced a significant, constitutive level of CAT activity. A dose-response curve, using 8 μg of OCAT/265 DNA, revealed maximum PMA induction of CAT activity at 50 ng/ml (data not shown).

To establish whether the transcriptional start site for the SAA-CAT gene construct is the same as the start site for the SAA gene, RNase mapping was performed on RNA isolated from cells transfected with both the whole gene and the OCAT/265 construct. The analyses revealed that transcription initiation occurred at exactly the same position when the whole SAA gene was used or when transcription was directed by just the 265-bp fragment on the CAT gene (Fig. 4).

Specific DNA-binding nuclear protein induced by PMA. Since (i) PMA was capable of inducing transcription of the complete SAA gene and (ii) a short 5′ region of the gene was capable of conferring inducibility to a heterologous reporter gene, DNA-binding proteins from PMA-treated cells were examined. We isolated total nuclear protein extracts from untreated and PMA-treated HeLa cells and assayed for DNA-binding activity by the DNA-binding gel shift assay. We found strong binding of nuclear proteins from the treated cells to the 265-bp 5′ fragment capable of conferring PMA responsiveness (Fig. 5A). Furthermore, this binding could be abolished by using 50 ng of unlabeled fragment as competitor. Binding could also be abolished by using an unlabeled HIV-1 LTR DNA fragment. This DNA fragment has been demonstrated to contain two binding sites for NFκB (32). DNA binding was not affected when a nonspecific competitor, a 465-bp SAAg9 intron 1 DNA fragment, was used. We also studied nuclear binding proteins from mouse L cells persistently transfected with SAAg9. Specific binding to the 265-bp fragment was found with extracts from PMA-treated cells, and again the binding could be abolished with 50 ng of the HIV-1 LTR fragment (Fig. 5A).

Specific binding of an NFκB-like DNA-binding protein. To delineate the exact binding site of the inducible NFκB-like DNA-binding protein, DNA methylation interference analysis was performed. Analysis of bound and unbound DNA strands spanning the 265-bp 5′ fragment revealed binding to

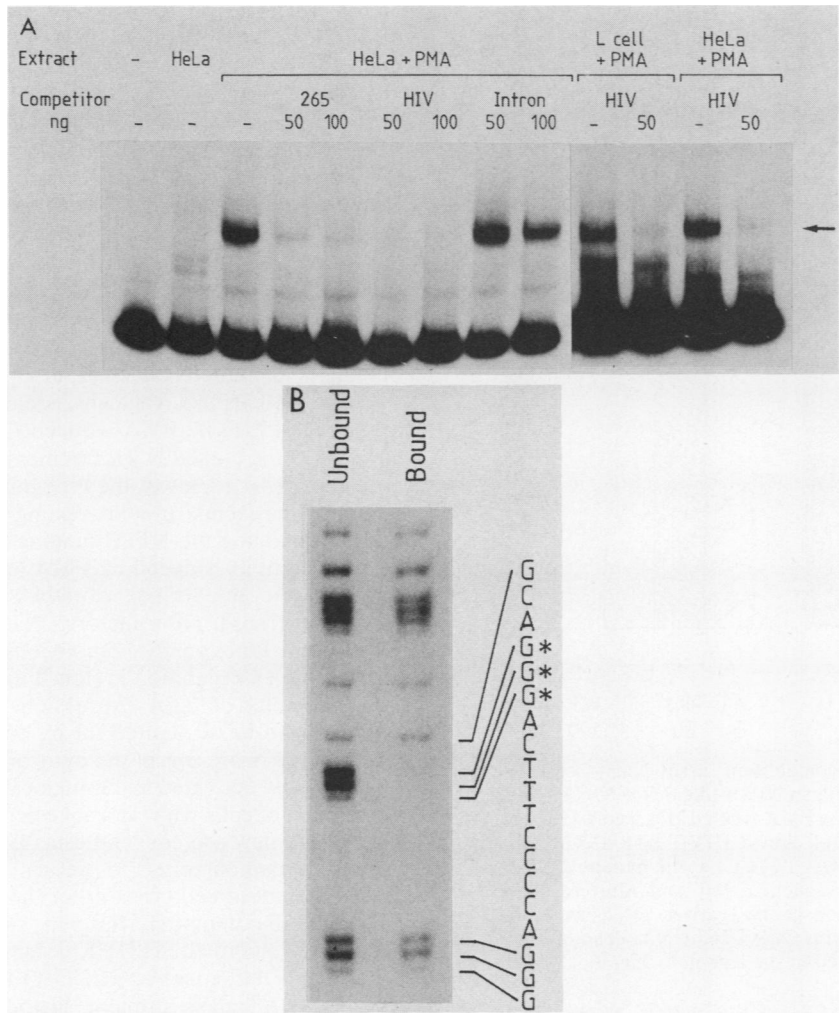

FIG. 5. DNA-binding assays demonstrating binding of an NFκB-like nuclear protein to the SAA 5′ region. (A) Nuclear extracts were isolated from HeLa cells and SAAg9-transfected L cells after PMA treatment (50 ng/ml for 4 h), and 5 μg of nuclear protein was incubated with 10^4 cpm of the ^{32}P-labeled 265-bp fragment for 15 to 30 min with or without unlabeled competitor DNA. Reaction products were electrophoresed through a nondenaturing 4% polyacrylamide gel, the gel was dried, and bands were visualized by autoradiography. Arrow indicates retarded band. Competitor DNAs: 265, 265-bp *Sau*3A 5′ SAA fragment from SAAg9 (Fig. 1A); HIV, 361-bp *Eco*RV-*Bgl*II fragment of HIV-1 LTR DNA; Intron, 465-bp *Ava*II intron 1 fragment of SAAg9. (B) The sequence of the protein-binding region was determined by methylation interference analysis. The 265-bp fragment was end labeled with ^{32}P, the labeled ends were separated by *Sma*I digestion, and the DNA was partially methylated at G residues by incubation with dimethyl sulfate. A large-scale DNA-binding gel electrophoresis assay was performed on both fragments, and the bound and unbound DNAs were eluted from the gel. The DNA was then cleaved by incubation with piperidine, and the resultant DNA fragments were separated by electrophoresis on a 12% polyacrylamide sequencing gel. Asterisks identify protected G residues.

a region of DNA containing the viral enhancer sequence GGGACTTTCC (Fig. 5B). This pattern of binding was similar to that described for the immunoglobulin enhancer binding factor NFκB, which is induced in HeLa cells by PMA.

Induction of CAT activity with phorbol ester by the 5′ SAA gene fragment is via binding of an NFκB-like factor. To establish whether the NFκB-like factor binding to the 5′ SAA region was responsible for PMA-induced CAT activity, we cotransfected the OCAT/265 DNA construct with a fragment from the HIV-1 LTR containing two copies of the wild-type or mutant NFκB-binding site. This mutant sequence was previously shown to abolish the binding of NFκB transcription factor (32). There was complete abolition of the PMA-inducible CAT activity with 50 μg of wild-type competitor DNA (Fig. 6). There was no competition when the cloned mutant NFκB-binding sequence was used.

DISCUSSION

The acute-phase response results in a dramatic change in the expression of a group of serum proteins. These changes can be induced in vivo and in vitro by treatment with cytokines individually or in combination. Examples include induction of SAA (39, 49), complement factors B (36, 39) and C3 (14, 20), AGP, and haptoglobin (20, 26, 39) by IL-1; induction of AGP (20, 38), fibrinogen, α1-antichymotrypsin, haptoglobin, cysteine protease inhibitor, and α2-macroglobulin (1, 20) by IL-6; induction of SAA (49), complement C3,

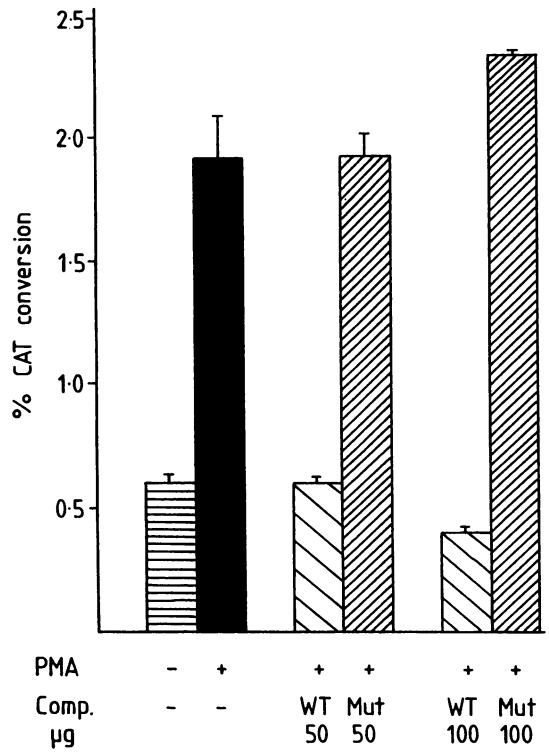

FIG. 6. Cotransfection-competition assay demonstrating that phorbol ester induction is via an NFκB-like factor. OCAT-265 DNA (1 μg) was cotransfected into PMA-treated HeLa cells (2.5 ng/ml for 2.5 h) with 50 and 100 μg of cloned HIV-1 LTR DNA containing either the wild-type ($^{A}_{G}$GGGACTTTCC) or the mutant ($^{A}_{G}$CTCACTT TCC) NFκB-binding site sequence (WT and Mut, respectively). Equivalent amounts of nonspecific carrier DNA were used in transfection experiments with OCAT/265 DNA alone. CAT assays were performed as described in the legend to Fig. 3.

haptoglobin, and AGP (7, 20, 26) by tumor necrosis factor; and induction of complement factors B and C2 by IFN-γ (47).

5′ *cis*-acting control regions have been identified in a number of cytokine-responsive acute-phase genes. These include the IL-1-responsive factor B, AGP, haptoglobin, and stromelysin genes (19, 34, 38; H. R. Colten, personal communication) and the IL-6-responsive C-reactive protein gene (3). We have characterized the 5′-flanking region of an SAA gene and identified a phorbol ester-responsive enhancer sequence that is activated by binding to an NFκB-like nuclear protein. It is not yet clear which cytokine(s) acts via this enhancer region in SAA expression. One candidate would be IL-1 because PMA can replace IL-1 in lymphocyte comitogenesis assays (48). Furthermore, recent experiments (L. Osborn, S. Kunkel, and G. Nabel, Proc. Natl. Acad. Sci. USA, in press) have shown that IL-1 can induce transcription of CAT directed by the HIV-1 LTR via an NFκB-like factor. Our experiments demonstrate that PMA induces a moderate 5-fold increase in SAA mRNA, whereas IL-1 produces a >50-fold increase (49). Therefore, PMA can mimic only part of the action of IL-1 in this system, and the 9-bp consensus sequence (Fig. 1B) found by sequence homology between the IL-1-inducible genes (SAA, factor B, AGP, stromelysin, and IL-6) may have functional importance.

A number of phorbol ester-inducible nuclear proteins have been identified. These include AP-1, AP-2, AP-3 (2, 10, 27), and NFκB (44). The binding site for one of these factors, NFκB, has been identified in a series of viral enhancers, including HIV, simian virus 40, and cytomegalovirus; it has also been found upstream of major histocompatibility complex class I genes and in the immunoglobulin κ light-chain gene enhancer. NFκB was found in activated B cells producing immunoglobulin κ light chain. However, it was also identified in cells that did not express the κ gene (HeLa and T cells), for which its target genes were unknown. Here we have demonstrated the probable involvement of NFκB in the transcription of another cellular gene, SAA. We have characterized a phorbol ester-inducible enhancer sequence from the 5′-flanking region of an SAA gene and demonstrated binding of an inducible nuclear factor to a sequence between −82 and −91 in this fragment, identical to the recognition sequence of NFκB. PMA-induced CAT activity was abolished by using cloned DNA fragments from the HIV-1 LTR containing two copies of the recognition sequence in transfection experiments. It is interesting that cloned oligonucleotides containing the NFκB-binding sequence did not abolish CAT activity induced by PMA (data not shown), which indicates the importance of DNA conformation for the binding of transcription factors. These results suggest that NFκB or a functionally homologous protein has a role in transcriptional regulation in non-B cells.

The rapidity of SAA expression in the acute-phase response could be accounted for by activation of this region, since NFκB is present in the cytosol in an inactive state (4) and is rapidly activated and translocated to the nucleus upon stimulation of cells with phorbol ester. This process does not require any new protein synthesis (45). However, NFκB has yet to be demonstrated in tissues that normally express SAA. Factors in cell types other than B, T, and HeLa that have DNA-binding properties very similar to those of NFκB have been identified. H2TF1, a factor found in most cell types, binds the sequence GGGGATTCCCC upstream of the mouse *H-2K*^{b} class I major histocompatibility gene (5). Similarly, the phytohemagglutinin- and PMA-inducible nuclear protein HIVEN86A binds to the sequence GGGGAA TCTCCC, upstream of the IL-2 receptor alpha gene (8, 28). NFκB also binds with high efficiency to both of these sequences (6, 8). Moreover, the PMA-inducible factor AP-3 is able to bind an identical sequence in simian virus 40 but is slightly different in binding characteristics from NFκB (10). Thus, several closely related protein factors are able to bind to subtly different 5′ enhancer sequences, and any of these may be the true physiological activator for SAA gene transcription.

Other DNA-binding proteins have been shown to activate some other acute-phase genes; e.g., hepatocyte-specific nuclear factor 1 (HNF1) activates the α- and β- fibrinogen and α_1-antitrypsin genes (12), major late transcription factor activates the rat γ-fibrinogen gene (11), and LF-A1 and LF-B1 (or HNF1) activate the α1-antitrypsin and haptoglobin genes (23). However, binding sequences for these proteins are absent in the SAA promoter region. Also, the SAA gene does not contain the heat shock consensus sequence (35) found in the promoter regions of the C-reactive protein and factor B genes. Thus, a complex pattern of interactions between different, readily activatable nuclear factors and *cis*-acting DNA sequences of different acute-phase genes is now emerging. Future comparison of wild-type and mutant SAA genes should test directly the importance of these factors in the physiology of the acute-phase response.

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